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Nitrogenase Activity and Regeneration of the Cellular ATP Pool in *Azotobacter vinelandii* Adapted to Different Oxygen Concentrations

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The in vivo activity of nitrogenase under aerobiosis was studied with diazotrophic chemostat cultures of *Azotobacter vinelandii* grown under glucose- or phosphate-limited conditions at different dilution rates (*D*s, representing the growth rate μ) and different dissolved oxygen concentrations. Under steady-state conditions, the concentration as well as the cellular level of ATP increased in glucose-limited cultures when *D* was increased. Irrespective of the type of growth limitation or the dissolved oxygen concentration, the steady-state concentrations of ATP and of dinitrogen fixed by nitrogenase increased in direct proportion to each other. Specific rates of dinitrogen fixation as well as of the regeneration of the cellular ATP pool were compared with specific rates of cellular respiration. With glucose-limited cultures, the rate of regeneration of the ATP pool and the rate of respiration varied in direct proportion to each other. This relationship, however, was dependent on the dissolved oxygen concentration. As compared to the phosphate-sufficient control, phosphate-limited cultures exhibited the same nitrogenase activity but significantly increased respiratory activities. Rates of ATP regeneration and of cellular respiration of phosphate-limited cultures did not fit into the relationship characteristic of glucose-limited cultures. However, a linear relationship between the rates of dinitrogen fixation and ATP regeneration was identified irrespective of the type of growth limitation and the dissolved oxygen concentration. The results suggest that the ATP supply rather than cellular oxygen consumption is of primary importance in keeping nitrogenase activity in aerobic cultures of *A. vinelandii*.

The biological fixation of dinitrogen depends on the activity of the highly oxygen sensitive nitrogenase enzyme complex (9, 11). In spite of this sensitivity, members of the diazotrophic azotobacters are able to grow under fully aerobic conditions. It was shown before that cells of *Azotobacter vinelandii* grown at different dissolved oxygen concentrations exhibited identical nitrogenase polypeptide contents and that ambient oxygen had no significant effect with respect to the turnover of these polypeptides (7). Protection of nitrogenase against oxygen damage has been proposed to occur in azotobacters on the basis of the following two mechanisms: (i) high respiratory activities that remove oxygen already at the surface of the cell and (ii) reversible conversion of the enzyme into a protected, yet-inactivated state (3, 4, 8, 33). The first hypothesis is believed to explain the function of nitrogenase when cells are growing diazotrophically in the presence of oxygen. The second mechanism is required to protect the reversibly inactivated enzyme from oxygen damage as soon as the first mechanism becomes overburdened by a sudden increase in the ambient oxygen concentration (oxygen stress) (8, 35). It has been suggested that inactivation (switch off) occurs when nitrogenase of *A. vinelandii* reaches a certain degree of oxidation, giving rise to the formation of an oxygen-stable complex of the enzyme with an FeSII protein (10, 27, 28, 33).

Although the hypothesis of respiratory protection has been generally accepted not only for azotobacters but also for other diazotrophs, a number of detailed investigations showed that removal of oxygen at the surface of the cell is not sufficient to explain the activity of nitrogenase under aerobic conditions. For example, it was shown with *A. vinelandii* growing at high dissolved oxygen concentrations that the respiratory activity does not significantly increase with an increase in the dissolved oxygen concentration (25). Moreover, cultures of *A. vinelandii* exhibited comparable cellular contents and nitrogenase activities in spite of considerable differences in cellular oxygen consumption (7, 13). Finally, it has been demonstrated that oxygen enters the cells of *A. vinelandii* without impairing the function of nitrogenase (21). Additional protection of nitrogenase may be provided by the decrease of the cellular surface area per cell volume (24) as well as by the increase of the cellular content of superoxide dismutase (6) occurring when the dissolved oxygen concentration of chemostat cultures of *A. vinelandii* is increased. Moreover, it has been proposed that nitrogenase stays active in aerobic cultures as long as the low redox potential necessary for its function is warranted by a sufficiently high flux of electrons through the enzyme complex (13). This is in accord with the hypothesis of autoprotection of nitrogenase, assuming that oxygen enters the cells where it is reduced by nitrogenase (31). Evidently, this type of protection can be effective only as long as the reduced state of nitrogenase is maintained, i.e., as long as nitrogenase activity is not switched off.

Reduction of nitrogenase requires both reducing equivalents and high amounts of MgATP. The importance of the latter in keeping nitrogenase active in the presence of oxygen became apparent when it was shown that reversible inactivation of nitrogenase under oxygen stress was accompanied by a reversible decrease in the cellular ATP pool (16). Moreover, it has been reported that adaptation from a low to a higher oxygen concentration was dependent on the recovery of the ATP pool from stress. Consequently, a mutant partially impaired in respiratory electron transport (12) and, thus, in energy regeneration is unable to grow diazotrophically at increased oxygen

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concentrations. These results suggest a quantitative relationship between the nitrogenase activity and the energy status of the cell. As a matter of fact, the present communication provides evidences for the dependence of nitrogenase activity on the rate at which the cellular ATP pool is regenerated. Since, in contrast to cellular respiration, this was independent of the respective growth conditions, it is concluded that ATP regeneration rather than oxygen consumption is of central importance in keeping nitrogenase active under aerobiosis.

MATERIALS AND METHODS

Organism and growth conditions. *A. vinelandii* wild-type strain OP (ATCC 13705) as well as the mutant strains MK5 (12, 16) and *hoxKG* (17) were grown diazotrophically in an oxygen- and pH-controlled chemostat. Dissolved oxygen concentrations were measured and kept constant as described previously (25). The cultures were grown in nitrogen-free medium (16, 25) with 15.1 mM D-glucose as the carbon source in glucose- and phosphate-limited cultures. Details of phosphate limitation are presented in Results. Limitation by glucose was confirmed as before by increasing the steady-state biomass level after increasing the supply of the carbon source (13). Glucose-limited cultures were grown with 0.8 g of K_2HPO_4 per liter and 0.2 g of KH_2PO_4 per liter, and phosphate-limited cultures were grown with either 8 mg of K_2HPO_4 per liter and 2 mg of KH_2PO_4 per liter or 4 mg of K_2HPO_4 per liter and 1 mg of KH_2PO_4 per liter.

Activity measurements. Specific rates of dinitrogen fixation by the cultures were calculated from total nitrogen contents at steady-state conditions, as described by Bühler et al. (2). In situ respiratory activities were estimated on the basis of the gas flow rate through the culture and the difference between oxygen concentrations at the gas inlet and outlet. Oxygen concentrations were determined by gas chromatography (17).

Determination of adenine nucleotide levels. To quantify ATP, ADP, and AMP contents, adenine nucleotides were extracted as described previously (16). Adenine nucleotides in the extracts were determined by published methods (16, 19, 34).

Quantitative determinations. Protein was determined by the method of Lowry et al. (18). Steady-state levels of glucose were determined on the basis of NADPH liberated in the course of the reaction with hexokinase and glucose-6-phosphate dehydrogenase (test combination for D-glucose purchased from Boehringer, Mannheim, Germany; detection limit, 0.2 mg/liter).

RESULTS

Glucose-limited cultures. Steady-state activities of cellular respiration as well as of nitrogenase of *A. vinelandii* may be varied by adjusting chemostat cultures to different dilution rates (*D*s). In addition, this culture system allows one to strictly control the dissolved oxygen concentration. On this basis, glucose-limited chemostat cultures of *A. vinelandii* were used to study, under steady-state conditions, possible relationships between in situ activities of cellular respiration and nitrogenase, on the one hand, and the cellular adenine nucleotide pool and its regeneration, on the other. Steady-state biomass (protein) levels depended in inverse proportion on the dissolved oxygen concentration (Fig. 1A). Increasing *D* from 0.05 to 0.32 h^{-1} led to linear increases in biomass levels when the cultures were adapted to either 135 or 68 μM O_2. With cultures adapted to 11.3 μM O_2, however, the biomass level slightly increased up to a *D* of about 0.15 h^{-1} and approached constancy at the higher *D* values. Determination of the glucose levels under steady-state conditions revealed that the cultures consumed essentially all of the limiting substrate under all of the conditions tested.

The activity of oxygen consumption by the cultures increased when the dissolved oxygen concentration was increased from 11.3 to 68 or 135 μM O_2. In addition, at a given dissolved oxygen concentration, oxygen consumption linearly increased with an increasing *D* (Fig. 1B). Specific rates of dinitrogen fixation were calculated on the basis of *D* and the total amounts of dinitrogen fixed by the cells under steady-state conditions (2). Since, under steady-state conditions, cells fixed 6.7 ± 0.4 μmol of dinitrogen per mg of protein irrespective of the dissolved oxygen concentration, the specific rate of dinitrogen fixation increased linearly with increasing *D*. A comparable result was shown before with cultures of *A. vinelandii* grown under conditions of either sucrose, citrate, or acetate limitation (13).

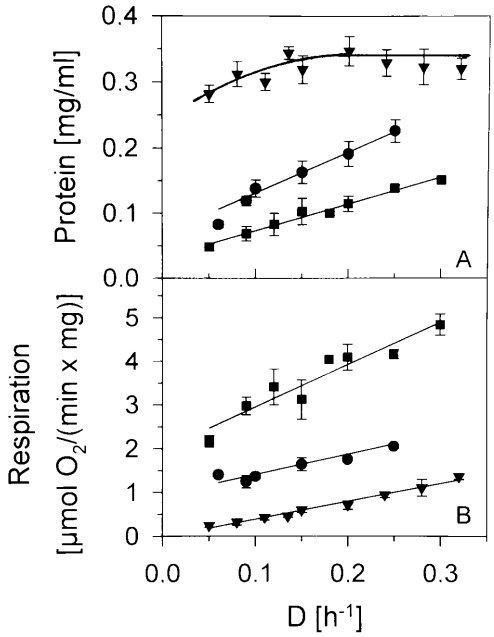

FIG. 1. Steady-state protein levels (A) and specific in situ respiratory activities (B) of glucose-limited chemostat cultures of *A. vinelandii* growing diazotrophically at different *D* values and at dissolved oxygen concentrations of 11.3 (▼), 68 (●), and 135 (■) μM.

Adenine nucleotide contents were determined after adaptation of the cultures to the different conditions described above (Fig. 1). Specific ATP content increased with increasing *D*, while ADP and AMP contents stayed largely constant. In Fig. 2 are depicted representative results obtained for a culture of *A. vinelandii* adapted to 135 μM O_2 and *D* values ranging from 0.05 to 0.30 h^{-1}.

Phosphate-limited cultures. Diazotrophic phosphate-limited continuous cultures of *Azotobacter chroococcum* have

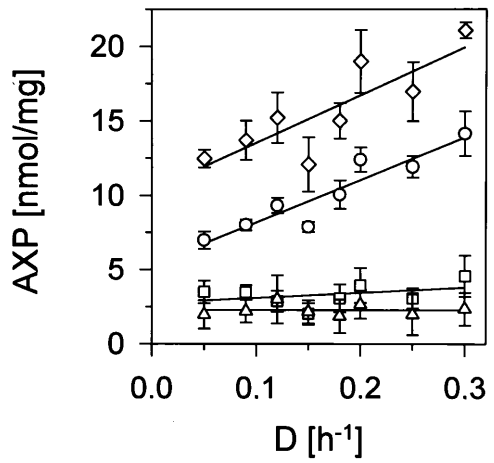

FIG. 2. Specific adenine nucleotide contents of glucose-limited chemostat cultures of *A. vinelandii* adapted to 135 μM O_2 and different *D* values. Symbols: ◇, sum of adenine nucleotides; ○, ATP; □, ADP; △, AMP.

TABLE 1. Steady-state protein levels, respiratory activities, and specific adenine nucleotide concentrations of phosphate-limited and phosphate-sufficient chemostat cultures of *A. vinelandii* growing diazotrophically at dissolved oxygen concentrations of 68 and 135 μM[a]

DOC (μM)	Phosphate (mg/liter)	Protein (mg/ml)	O_2 respired (μmol min^{-1} mg^{-1})	Adenine nucleotide concn (nmol/mg)			
				ATP	ADP	AMP	AXP
68	580	0.14 ± 0.01	1.37 ± 0.05	11.8 ± 0.7	4.3 ± 1.3	2.0 ± 1.0	18.1 ± 1.4
68	5.8	0.07 ± 0.01	2.81 ± 0.23	8.4 ± 0.3	2.1 ± 0.3	1.6 ± 0.8	12.1 ± 0.6
68	2.9	0.04 ± 0.00	3.97 ± 0.53	6.5 ± 0.2	2.3 ± 0.7	1.6 ± 0.8	10.4 ± 0.7
135	580	0.08 ± 0.01	2.85 ± 0.21	8.1 ± 0.3	3.0 ± 0.5	2.2 ± 0.7	13.3 ± 1.0
135	5.8	0.05 ± 0.00	4.30 ± 0.08	8.2 ± 0.5	1.8 ± 0.2	1.2 ± 0.4	11.2 ± 0.7

[a] Phosphate-limited cultures were grown with 5.8 or 2.9 mg of phosphate per liter; phosphate-sufficient cultures were grown with 580 mg of phosphate per liter. The *D* in all cultures was 0.1 h^{-1} with 15.1 mM glucose in the inflowing medium. All of the cultures fixed dinitrogen at a rate of 10.9 nmol min^{-1} mg^{-1}. DOC, dissolved oxygen concentration; AXP, sum of adenine nucleotides. Values for protein levels, respiratory activities, and adenine nucleotide concentrations are means ± standard errors of the means.

been described as extremely oxygen sensitive (3, 4, 15). Investigations of batch cultures of *A. vinelandii* grown at low phosphate concentrations revealed lower respiratory activities and decreased ATP contents as well as energy charge values than those of cultures grown at saturating phosphate concentrations (5, 32). Since some of the results obtained with phosphate-limited cultures were proposed to support the hypothesis of respiratory protection (3, 4, 15), we included phosphate limitation in the present study as well. Phosphate-limited cultures of *A. vinelandii* were grown at a *D* of 0.1 h^{-1} and at either 68 or 135 μM O_2. After adaptation to 68 μM O_2, steady-state protein levels of the cultures grown with 5.8 or 2.9 mg of phosphate per liter were significantly decreased compared with that of glucose-limited cultures containing the saturating phosphate concentration of 580 mg per liter of growth medium (Table 1). These results proved that at the two lower phosphate concentrations, biomass production was phosphate limited. Unexpectedly, the rates of in situ oxygen consumption by these phosphate-limited cultures were increased by factors of 2 and 3 at 5.8 and 2.9 mg of phosphate per liter, respectively, compared with that of the control. Stimulation of the rate of oxygen consumption by phosphate limitation was also observed with cultures adapted to 135 μM O_2. Irrespective of the type of limitation and the dissolved oxygen concentration, however, all of the cultures fixed dinitrogen at a rate of 10.9 nmol per min per mg of protein when growing at a *D* of 0.1 h^{-1} (Table 1). At both oxygen concentrations employed, the sum of the three adenine nucleotides was lower in phosphate- than in glucose-limited cultures. A significant effect of phosphate limitation on the value of the energy charge could not be observed.

Steady-state concentrations of ATP and fixed dinitrogen. ATP concentrations of the cultures described above were plotted versus the corresponding concentrations of dinitrogen by fixed nitrogenase (Fig. 3). In addition, representative data which were obtained with mutant MK5 lacking cytochrome *d* and, thus, the uncoupled branch of the respiratory chain (12, 16) as well as with mutant *hoxKG* lacking the uptake hydrogenase (17) were included. Linear regression analysis of all of the data ($n = 30$) yielded a straight line based on a significance level of >95% (correlation coefficient [*r*], 0.9829). It should be noted that this result was independent of the strain employed, the type of growth limitation (glucose or phosphate), or the dissolved oxygen concentration.

Regeneration of the cellular ATP pool and cellular activities. The specific rate at which the cellular ATP level was regenerated was calculated by multiplying the specific ATP content by the corresponding *D* (i.e., μ) value. On this basis, it becomes possible to evaluate relationships between the regeneration of the cellular ATP pool and cellular activities, which respectively utilize and regenerate ATP like the activity of dinitrogen fixation or cellular respiration. When the specific rates of ATP regeneration were plotted versus the corresponding activities of cellular oxygen consumption, relationships between both parameters were obtained which were linear and yet confined to each of the three dissolved oxygen concentrations investigated (Fig. 4). Respiratory rates determined with phosphate-limited cultures, however, did not fit into any of these relationships.

On the other hand, if the rates of ATP regeneration were plotted versus the corresponding rates of dinitrogen fixation, a linear relationship that accommodated all of the results independent of the dissolved oxygen concentrations employed became apparent (Fig. 5). Importantly, the results obtained with mutants MK5 and *hoxKG* as well as those obtained with phosphate-limited cultures fit into this relationship, too. Regression analysis of the data ($n = 30$, $r = 0.9743$) revealed that 95% of the variation in the amount of nitrogen fixed is due to the variation in the regeneration rate of the ATP pool. It should be noted, however, that the statistical treatment of the data yielded a straight line which extrapolates to a rate of dinitrogen fixation above zero. Since dinitrogen fixation at zero ATP regeneration appears physiologically questionable, it may be postulated that, at the lowest rates, the relationship deviates from linearity so that the rates of dinitrogen fixation and of

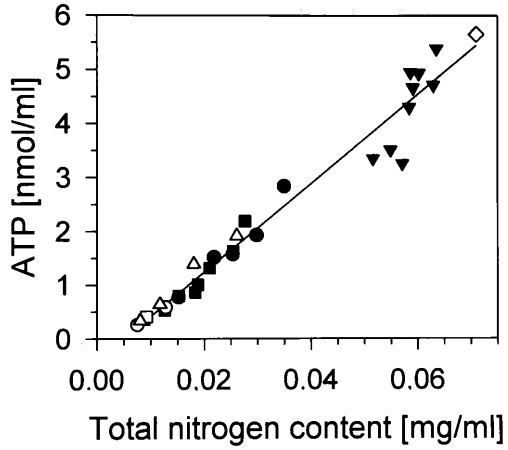

FIG. 3. Relationship between the steady-state concentrations of ATP and dinitrogen fixed by nitrogenase. The values were obtained with glucose- and phosphate-limited chemostat cultures of *A. vinelandii* growing diazotrophically at different *D* values and dissolved oxygen concentrations. Glucose-limited cultures contained dissolved oxygen concentrations of 11.3 (▼), 68 (●), and 135 (■) μM; phosphate-limited cultures contained concentrations of 68 (○) and 135 (□) μM O_2. Representative values obtained with mutant MK5 (16) at 2.3 μM O_2 (◇) and mutant *hoxKG* (17) at 135 μM O_2 (△) are also included.

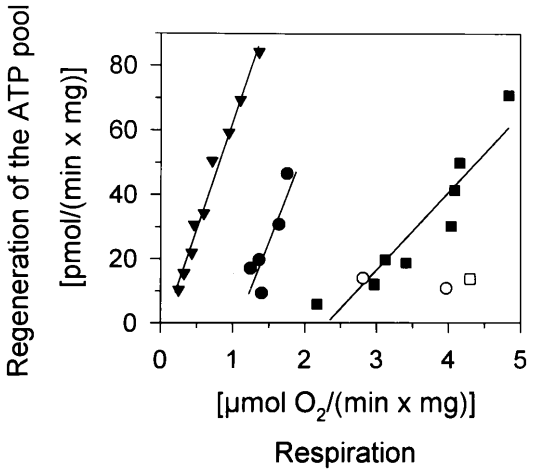

FIG. 4. Relationship between the specific rate of regeneration of the ATP pool and the specific respiratory activity. The values were obtained with glucose- and phosphate-limited chemostat cultures of *A. vinelandii* growing diazotrophically at different *D* values and dissolved oxygen concentrations. Glucose-limited cultures had dissolved oxygen concentrations of 11.3 (▼), 68 (●), and 135 (■) μM; phosphate-limited cultures contained 68 (○) and 135 (□) μM O_2.

ATP regeneration concomitantly approach zero. The lowest values obtained at the three dissolved oxygen concentrations support this presumption.

From the data illustrated in Fig. 4 and 5, it can be concluded that with respect to each of the three oxygen concentrations, the rate of dinitrogen fixation by glucose-limited cultures increased in direct proportion to the rate of oxygen consumption. Again, the results obtained with phosphate-limited cultures did not fit into any of these relationships. In other words, the observed relationship between the rates of oxygen consumption and dinitrogen fixation depended on the growth conditions.

DISCUSSION

In chemostat cultures of *A. vinelandii* limited by an organic substrate like glucose, the substrate presents the only source of carbon as well as the only source of reducing equivalents and energy. Since cells of *A. vinelandii* obviously can afford to completely dissimilate a high proportion of the limiting substrate (1), it may be assumed that carbon limitation actually means energy limitation. In the present study, we determined steady-state levels of adenine nucleotides and, in particular, the level of the growth-limiting ATP. To interpret the results, it should be remembered that according to the theory of continuous chemostat cultures, the steady-state concentration of the limiting substrate (S) is related to the steady-state level of biomass (X = protein or fixed dinitrogen) and the growth rate (μ) by the following equations: $X = Y(S_R - S)$ and $\mu = \mu_{max} S/(K_s + S)$, where S_R represents the concentration of the limiting substrate in the inflowing medium, μ_{max} is the maximal growth rate, and K_s is the substrate concentration required to grow at a rate of 1/2 μ_{max}, and Y is the growth yield coefficient. Importantly, every cellular parameter multiplies by μ to attain its steady-state level. This means that multiplication of steady-state nitrogen and ATP levels by μ yields the rate of in situ dinitrogen fixation and the rate at which the ATP pool is regenerated under culture conditions, respectively. When evaluating Y, it should be remembered that its value may be affected by maintenance processes as well as by energy loss and energy-spilling reactions, which utilize the limiting substrate but do not quantitatively add to biomass accumulation (22, 26, 30). In diazotrophic organisms and particularly in aerobic azotobacters, the high energy requirement of nitrogenase contributes significantly to the high maintenance activities known for these organisms (16, 29). It was reported before that increases in the dissolved oxygen concentration were accompanied by increases in the maintenance requirements of diazotrophic cultures of *A. vinelandii* and decreases in the Y values (14, 17). Importantly, however, it was also reported that the true growth yield (Y^G) remained constant in cultures grown at different dissolved oxygen concentrations and carbon sources. This indicates that energy regeneration from glucose was constant.

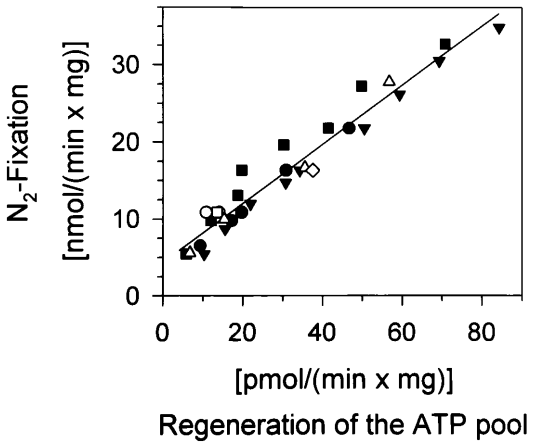

FIG. 5. Relationship between the specific rate of nitrogen fixation and the specific rate of regeneration of the ATP pool. For further details and definition of symbols, see the legend to Fig. 3.

As shown with *Azotobacter beijerinckii* (20), the steady-state level of the limiting ATP increased when D was increased. Interpretation of these results in light of the theory of continuous cultures leads to the following conclusions. Since diazotrophic cultures can be considered energy limited and since the concentration of the energy source in the feed (S_R) was constant, it follows that the observed D-dependent increases in the steady-state levels of both S (ATP) and X were due to an increase in the efficiency of energy utilization (Y_{ATP}) for biomass formation, i.e., dinitrogen fixation. Augmentation of Y may be explained by the fact that, upon increasing the substrate supply with increasing D, the relative influence of maintenance functions on Y decreases (17, 22). In diazotrophic cultures of *A. vinelandii*, the unusually high maintenance activities with glucose and other carbon sources vary largely in direct proportion to the oxygen concentration (14, 17). Therefore, when increasing D at the higher oxygen concentrations, the relative influence of maintenance activities on Y does not decrease in *A. vinelandii* to a negligibly low level as it does in other bacteria (22). This explains why the herein described glucose-limited diazotrophic cultures adapted to a fairly low dissolved oxygen concentration of 11.3 μM did reach the maximal biomass level at the higher D values while cultures adapted to 68 and 135 μM O_2 did not.

Phosphate-limited diazotrophic cultures of azotobacters were reported to exhibit increased oxygen sensitivity (3, 4, 15). In light of the hypothesis of respiratory protection, this was suggested to result from presumed lower rates of oxygen consumption (3). The present investigation, however, shows the opposite, namely, that phosphate-limited cultures of *A. vinelandii* exhibited significantly higher rates of respiration. Nevertheless, the specific activity of cellular nitrogenase remained

unaffected. Thus, our present results obtained with strictly controlled phosphate-limited chemostat cultures do not support previous data included in the formulation of the hypothesis of respiratory protection of nitrogenase (3, 4).

However, the results of the present investigation are in accord with an alternative hypothesis explaining the function of nitrogenase despite the presence of oxygen. This hypothesis postulates that reduction of nitrogenase and, thus, its function at ambient oxygen concentrations are warranted as long as the enzyme is supplied with sufficiently high amounts of ATP (13, 16). In agreement with this hypothesis and irrespective of the oxygen concentration and the type of growth limitation, uniform relationships became apparent either when the size of the ATP pool was plotted versus the concentrations of dinitrogen fixed by nitrogenase or when the specific rates of regeneration of the ATP pool were plotted versus the specific rates of dinitrogen fixation. This, however, was not the case when the rates of ATP regeneration by glucose-limited cultures were plotted versus the corresponding rates of oxygen consumption, and this discrepancy became even more pronounced with phosphate-limited cultures.

So far, the discussion of the present data has concentrated on the relationship of the activity of nitrogenase and its energy requirement. Since, however, nitrogenase formation and turnover may be assumed to utilize a significant proportion of the cellular energy pool, it might be postulated that the ATP pool is limiting these two processes rather than the activity. This possibility can be excluded by previous findings showing that neither the cellular level nor the turnover of the nitrogenase polypeptides of *A. vinelandii* is affected by oxygen (7). Moreover, since it was shown that the cellular level of both nitrogenase polypeptides is largely independent of *D*, it was concluded that the *D*-dependent increase of the cellular nitrogenase activity is regulated by the supply of reducing equivalents and energy (7). The present investigation confirms the latter mechanism.

In conclusion, the results of the present investigation suggest that the energy supply is a major factor in keeping nitrogenase active in aerobically growing *A. vinelandii*. In a sense, this expenditure of ATP to maintain the functionality of nitrogenase is, from an energetic point of view, a futile cycle (26). These conclusions lead to the final question: does this result apply to other diazotrophs, too? Previously, we showed that with *Rhodobacter capsulatus*, growing photoheterophically with saturating light, the rate of dinitrogen fixation is limited by the supply of reducing equivalents rather than by the supply of energy (23). This discrepancy can be explained by the fact that reduction of nitrogenase and its activity may be limited either by the supply of reducing equivalents or by the supply of energy. While *A. vinelandii* provides an example of the latter of the two possibilities, *R. capsulatus* growing energy sufficient provides an example of the former.

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